Sex pheromone of the tsetse species, *Glossina austeni*: isolation and identification of natural hydrocarbons, and bioassay of synthesized compounds

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Abstract. Copulatory responses of male *Glossina austeni* (Newstead) (Diptera: Glossinidae), that were elicited after contact with frozen female tsetse, were not observed after solvent washing of cuticular lipids. Chromatographic analysis of extracts from laboratory-reared and field-collected *G. austeni* females yielded natural hydrocarbons that were highly stimulatory to males. Most of this activity was produced by compounds in the alkene fraction. Gas chromatograms (GC) contained five natural alkenes; these were separated by preparative GC for bioassays conducted in Tanzania. The two major alkenes were identified using gas chromatography–mass spectrometry (GC–MS) to be 13,17-dimethyltritriacont-1-ene and 13,17-dimethylpentatriacont-1-ene, after the samples had undergone derivatization using dimethyl disulphide and saturation with deuterium. These alkenes and natural alkanes were quantified from *G. austeni* of both sexes from laboratory and field samples to confirm that their presence was consistent in this species. Trials of synthetic samples resulted in the order of biological activity for the stereoisomers of 13,17-dimethyltritriacont-1-ene as follows: *S,R*-33:1 > *R,S*-33:1 > *S,S*-33:1 > *R,R*-33:1. Dose–response data showed an ED_{50} at 5 μg per treated, solvent-washed male decoy. Of the four stereoisomers of 13,17-dimethylpentatriacont-1-ene, *R,R*-35:1 showed the most activity. This is the first report of alkene-induced sexual activity in males of the genus *Glossina*.

Key words. *Glossina austeni*, sex pheromone, mate recognition, Africa.

Introduction

Tsetse flies of 26 species and subspecies synthesize long-chain methyl-branched alkanes (Carlson *et al.*, 1993; Sutton & Carlson, 1997). Some of these components were found to be female-produced contact sex pheromones when synthetic analogs were tested at 1–20 μg. The first synthetic pheromone that produced potent stimulatory activity for conspecific male *Glossina morsitans morsitans* Westwood

(Langley *et al.*, 1975) was the trimethyl-branched 37 carbon-chain alkane, and lesser activity was observed with two dimethyl-branched homologs (Carlson *et al.*, 1978).

Female *G. pallidipes* Austen produced two dimethyl 35 carbon-chain isomers that stimulated conspecific males, and both showed biological activity as racemic mixtures (Carlson *et al.*, 1984). The most active *R,S-meso*-isomer of 13,23-dimethylpentatriacontane (Kuwahara & Mori, 1983) yielded biological activity with laboratory males at 4.5 μg (ED_{50}, effective dose at which 50% of the males responded fully), whereas the stereoisomeric mixture required twice as much material (McDowell *et al.*, 1985).

A sex pheromone present in extracts of female *G. palpalis palpalis* Rob-Des produced copulatory activity in males

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(Offor *et al.*, 1981), and a synthetic trimethyl-branched C35 alkane showed stimulatory activity (D. A. Carlson, unpublished data). A sex pheromone that released copulatory activity in male *G. tachinoides* (Westwood) was isolated from extracted female hydrocarbons. Bioactivity was observed in two natural dimethylalkane 37 carbon-chain isomers (Nelson *et al.*, 1988). Bioassays of both synthetic stereoisomeric mixtures showed slightly more activity in the 11,23- than the 13,25-dimethyl isomer (Carlson *et al.*, 1998). Recently the extracted hydrocarbons of *G. brevipalpis* (Newstead) females showed stimulatory activity in males, and these were shown to contain tetramethyl-branched 32 carbon alkanes not found in males (D. A. Carlson, unpublished data). In the studies with tsetse mentioned above, males of all species showed responses to the di- or trimethylalkane compounds only, and no responses were observed to separated alkanes containing just one methyl branch.

In the first report on *G. austeni* Newstead, 2-methyltriacontane (KI 3065, 47%) (Kovats Retention Indices; Kovats, 1965) and 2-methylhentriacontane (KI 3165, 34%) were found to be major components in males, with the former as the major component in females (69%, 3.5 µg). Both 9,15- and 11,15-dimethylnonacosanes (KI 2955) were found in males only (Nelson & Carlson, 1986). The 11,15-dimethylhentriacontane (KI 3155) was found only in males (34% of total), but the 11,15- and 13,17-isomers were found in both sexes. Three isomers, 11,15-, 13,17- and 15,19-dimethyltriatriacontane (KI 3355), were found in females as minor components (5%) with the latter not present in males, whereas 13,17- and 15,19-dimethylpentatriacontanes (KI 3555, 4%) were found in females only. Minor amounts of 5-methylene interrupted heptatriacontanes (11,17-, 13,19- and 15,21-dimethylheptatriacontanes) were found at KI 3755. Minor amounts of trimethylalkanes were present that had 3,3-, 5,3-, 3,5- and 7,3-sequences of methyl branching.

In the earliest study of alkenes of *G. austeni* by gas chromatography (GC), alkenes eluted slightly before alkanes with the same methyl branch position. Males contained KI 2950 and KI 3150 at 21% and 60%, respectively, with little of these present in virgin females. In virgin females, equal quantities of alkenes at KI 3350 and 3550 were present at 32% each (2.9 µg of each per female) (Carlson & Langley, 1986), but minor amounts of these were found in males. Alkenes were among the materials transferred between the sexes during mating, particularly the previously unidentified, major, male-produced KI 3150 alkene that was found on mated but not virgin females (Carlson & Langley, 1986).

Bioassays using male *G. austeni* from colonies in Bristol, U.K., showed very persistent sexual responses to 1–20 µg of synthetic 15,19-dimethyltritriacontane (Huyton *et al.*, 1980). Similarly, the total hydrocarbons from females, including alkenes, were stimulatory. However after separation, the natural 2-methylalkanes (KI 2965, 3065), dimethylalkanes (KI 3355, 3555, Nelson & Carlson, 1986), and even smaller amount of alkenes had lost all biological activity (P. A. Langley, unpublished data). Subsequent

bioassays of the natural alkanes included tests of three preparative collections of the major dimethyl alkanes, and included compounds that eluted before or with KI 3155, 3355 and 3555. We remained puzzled by those results that showed little consistent activity, unlike that obtained with the synthetic 15,19-dimethyltritriacontane, and recombination of material did not improve the results (P. A. Langley, unpublished data).

We now report the isolation and identification by gas chromatography–mass spectrometry (GC–MS) of dimethylalkenes found in females of *G. austeni*, and the first demonstration of stimulatory activity in conspecific males with the natural alkenes and synthesized stereoisomers of these unusual compounds.

Materials and methods

Biological samples

Samples of *G. austeni* were obtained from the International Atomic Energy Agency, Entomology colony at Seibersdorf, Austria, from the Tsetse Research Institute, Tanga, Tanzania and wild flies from South Africa (Hellsgate, Natal). Older samples were obtained from the original laboratory colonies in Bristol, U.K. (1982, courtesy of P. A. Langley) and Maison-Alfort, France, and museum specimens came from the Arabuka Forest (6 miles west of Kilifi, 1948) in coastal Kenya, and Mozambique (Muabsa).

Chemical separations and sample preparation

Female flies were frozen, then pinned and tested in small hand-held vials against individual mature 7–10-day-old males that had been fed blood 7–10 h previously (Carlson *et al.*, 1989). Large samples of dried female flies were extracted with *n*-hexane (Baxter-Burdick & Jackson, Muskegon, MI, U.S.A.) using 0.3 mL per fly, and the hydrocarbon fraction of each sample was separated from other lipids by column chromatography on silica gel. Subsequent argentation chromatography on 15% silver nitrate-silica gel of the hydrocarbon fractions obtained alkane and alkene fractions (Carlson & Langley, 1986). GC analyses were conducted (Nelson & Carlson, 1986) on these fractions to ensure that appropriate quantities were present for bioassays. Dimethyl disulphide addition and deuteration of the alkene fraction was performed as described (Carlson *et al.*, 2000).

Gas chromatography and gas chromatography/mass spectrometry

Cuticular hydrocarbons were quantified by GC utilizing a fused-silica capillary column (30 m × 0.32 mm i.d., 0.25 µm DB-1 stationary phase, J & W Scientific, Folsom, CA, U.S.A.) fitted to a Hewlett-Packard Model 6890 GC fitted with a cool on-column injector (OCI-3), and a

flame-ionization detector. Hydrogen was used as the carrier gas at a linear flow velocity of 40 cm/s. Each sample was reconstituted in 10 or 50 µL of hexane, as necessary and 1–2 µL aliquots were injected at 60°C. Each GC run was temperature-programmed as follows: hold for 2 min (60°C), ramp 20°C/min (60–230°C), ramp 2°C/min to 320°C, and hold at 320°C to elute all components (10 min). A PC-based data system, Turbochrom 3 (Perkin-Elmer Nelson, Cupertino, CA, U.S.A.) was used for data recording and quantification of GC peaks. Manual adjustment of some small peaks was required for consistent integration, and peaks below a threshold of 0.02% were not considered.

Electron ionization (EI) mass spectra were obtained using a Hewlett-Packard 5988A mass spectrometer interfaced to a HP 5890 GC fitted with an OCI-3 injector. Helium carrier gas at 9 psig gave a linear velocity of 40 cm/s with a DB-1 column as above, fitted with a retention gap (5 m × 0.53 mm i.d. deactivated, uncoated capillary column). The temperature program for GC/MS was: injection at 60°C (hold 2 min), ramp at 10°C/min to 220°C, ramp at 3°C from 220°C to 310° (hold 12–42 min). The mass spectrometer interface was maintained at 310°C, electron voltage at 70 eV, and the system parameters manually optimized to enhance the EI spectra in the critical region of m/z 200 to m/z 500. The mass spectral scan range extended from m/z 50 to m/z 700 with a scan rate (system limited) of 1.8 s per scan. Kovats retention Indices (KI) were determined by injection of a normal hydrocarbon standard containing alkanes of 9–36 carbons in length plus 15,19,23-trimethylheptatriacontane (KI 3770). KI retention index assignments were made in which KI 3100 represents a normal alkane of 31 carbons chain length, KI 3165 represents a 2-methyl-branched alkane, and KI 3155 represents an internally branched dimethyl alkane of the same carbon-chain length. The assignment of KI narrows the range of possible methyl-branch configurations in cases of overlapping peaks or insufficient EI spectra (Carlson *et al.*, 1998). The identification of methylalkanes followed the interpretations established previously for EI mass spectra (Nelson & Carlson, 1986; Nelson *et al.*, 1988).

Bioassays

Bioassays of dead and solvent-washed females were performed using flies from an original sample of 200 Seibersdorf females during studies conducted on separated hydrocarbons, alkanes, and alkenes in Seibersdorf in 1994. Following this, the extracted lipids, total hydrocarbons, alkane fraction and an alkene fraction from 2000 Seibersdorf females were bioassayed with 6-day-old laboratory males in Tanga. Individual male flies were held for several hours with a hexane-washed pinned dead female fly decoy in a small glass tube (3 × 5 cm). The males were exposed to the equivalent of lipids from 1 to 20 females from each fraction. The lipids were applied to wingless, dead, solvent-washed, male decoys, with tests of unwashed, dead females as positive controls. Dose–behavioural

response was recorded to yield the response of each male to treated decoys using the following scoring method: (1) no response despite brief contact with decoy; (2) mounting the decoy; (3) orientation to the copulatory position with abdomen curving; (4) full copulatory response including hypopygium extension, with 10 or six males at each dose. Data were analysed separately for alkanes and alkenes by comparing male responses to compounds across doses within peaks and again by comparing responses across compounds within doses. Analysis was done using the General Linear Models Procedure of the Statistical Analysis System; differences among means were evaluated by Duncan's means separation method ($P = 0.05$, SAS, 1992). The synthetic compounds were diluted with hexane and tested in the same manner by applying specifically measured quantities to the decoys.

Results

Identification of hydrocarbons

Alkanes. For the flies studied in this work (Table 1) and expanding upon work reported previously in Table 1 of Carlson *et al.* (2000), to which data for male flies have been added, slightly less of the 2-methyl alkanes such as KI 2865, 2965 and 3065 were found in females compared to previous results (Nelson & Carlson, 1986). The alkane quantities were distributed differently in the present GC–MS samples; there were larger amounts of alkanes present per female than in the original studies using Bristol flies. The major alkanes comprised 11,15- and 13,17-dimethylhentriacontanes (KI 3155), 11,15-, 13,17- and a small proportion of 15,19-dimethyltritriacontane (KI 3355), and 11,15-, 13,17- and a small proportion of 15,19-dimethylpentatriacontanes (KI 3555). In addition, there were small amounts of trimethylalkanes eluting just after each dimethylalkane peak, all of which had 3-methylene interrupted methyl branching, i.e., 13,17,21-trimethyltritriacontane (KI 3370), 13,17,21-pentatriacontane (KI 3570) and 13,17,21-trimethylheptatriacontane (KI 3770).

Wild males possessed about 3–10 times less of the longer chain alkanes than females collected at the same locations. Less information can be implied from the small but real quantities present on laboratory males, because these caged males had experienced extended contact with females.

Alkenes. Alkene patterns from wild flies collected in South Africa and Kenya in 1948 and laboratory flies from Austria are shown in Table 2. All males showed a consistent pattern of a major alkene at KI 3150 at 0.3–0.8 µg/male, and with the alkenes at KI 3350 and 3550 much reduced in wild males, but prominent in mated colony males at 0.28 µg/male. Wild Kenya females and mated females from Austria had KI 3150 as the major alkene (0.4 µg/female), and nearly the same quantity of peaks at KI 3350 and KI 3550 (0.25 µg/female of each). Wild females from South Africa and unmated females

Table 1. Cuticular alkanes from gas chromatography analysis of individual female and male *Glossina austeni* (μg/fly ± SD)

	Alkane											
	KI 2865	KI 2965	KI 3065	KI 3155	KI 3165	KI 3265	KI 3355	KI 3370	KI 3555	KI 3570	KI 3750	KI 3770
Females												
Mozambique: wild (*n* = 5)												
Mean	0.02	0.01	0.16	0.03	0.07	0.19	0.05	0.06	0.09	0.19	0.05	0.05
SD	0.004	0.009	0.015	0.040	0.042	0.113	0.026	0.027	0.050	0.030	0.007	0.002
South Africa: wild (*n* = 10)												
Mean	0.04	0.03	0.13	0.02	0.05	0.06	0.10	0.10	0.14	0.27	0.02	0.05
SD	0.015	0.007	0.039	0.007	0.019	0.016	0.018	0.023	0.035	0.016	0.003	0.005
Kenya: wild (*n* = 5)												
Mean	0.02	0.03	0.14	0.03	0.05	0.07	0.06	0.09	0.22	0.19	0.03	0.06
SD	0.009	0.011	0.041	0.009	0.035	0.069	0.044	0.088	0.021	0.059	0.017	0.009
Laboratory (Austria): mated (*n* = 5)												
Mean	0.04	0.10	0.25	0.05	0.13	0.11	0.10	0.06	0.08	0.06	0.01	0.01
SD	0.014	0.030	0.029	0.014	0.052	0.027	0.013	0.010	0.008	0.006	0.003	0.003
Laboratory (Austria): virgin (*n* = 5)												
Mean	0.04	0.08	0.35	0.05	0.06	0.11	0.08	0.05	0.08	0.05	0.02	0.01
SD	0.001	0.003	0.001	0.003	0.009	0.009	0.003	0.003	0.003	0.007	0.005	0.001
Males												
Mozambique: wild (*n* = 5)												
Mean	0.06	0.12	0.34	0.11	0.13	0.06	0.03	0.01	0.06	0.04	0.03	0.01
SD	0.022	0.078	0.073	0.041	0.017	0.040	0.018	0.013	0.021	0.027	0.025	0.012
South Africa: wild (*n* = 5)												
Mean	0.06	0.16	0.40	0.08	0.11	0.07	0.03	0.02	0.03	0.02	0.01	0.02
SD	0.022	0.084	0.054	0.034	0.027	0.032	0.025	0.013	0.020	0.009	0.003	0.002
Kenya: wild (*n* = 5)												
Mean	0.04	0.14	0.40	0.08	0.13	0.10	0.01	0.02	0.02	0.04	0.01	0.02
SD	0.011	0.115	0.078	0.022	0.013	0.006	0.009	0.012	0.025	0.013	0.003	0.008
Laboratory (Austria): mated (*n* = 5)												
Mean	0.07	0.17	0.40	0.06	0.12	0.03	0.03	0.02	0.02	0.04	0.01	0.01
SD	0.015	0.019	0.025	0.040	0.051	0.005	0.035	0.015	0.006	0.048	0.002	0.003
Laboratory (Austria): virgin (*n* = 5)												
Mean	0.05	0.11	0.39	0.04	0.09	0.10	0.06	0.05	0.04	0.05	0.01	0.01
SD	0.013	0.062	0.033	0.009	0.035	0.049	0.025	0.025	0.030	0.021	0.008	0.004

Table 2. Cuticular alkenes from gas chromatography analysis of *Glossina austeni* from different countries

	Kovats Retention Indices (KI) (mean μg/fly ± SD)					
Locality (*n*)	KI 2750	KI 2950	KI 3150	KI 3350	KI 3550	KI 3750
Males						
Kenya *(*10)	0.01 ± 0.005	0.07 ± 0.020	0.85 ± 0.038	0.04 ± 0.012	0.02 ± 0.011	0.01 ± 0.002
South Africa (5)	0.00 ± 0.005	0.03 ± 0.036	0.30 ± 0.48	0.02 ± 0.022	0.01 ± 0.011	0.00 ± 0.003
Laboratory (1250)*	0.01	0.07	0.54	0.28	0.09	0.01
Females						
Kenya *(*5)	0.02 ± 0.012	0.04 ± 0.010	0.39 ± 0.127	0.25 ± 0.127	0.27 ± 0.054	0.04 ± 0.045
South Africa (5)	0.01 ± 0.011	0.04 ± 0.010	0.22 ± 0.172	0.37 ± 0.163	0.34 ± 0.036	0.00 ± 0.031
Laboratory (2)†	0.02	0.06	0.22	0.34	0.35	0.01
Laboratory (2)‡	0.08	0.07	0.40	0.22	0.22	0.00

*Pooled sample from IAEA Austria colony. Kenya and South Africa specimens were wild.
†Uninseminated pool of two flies from IAEA colony.
‡Mated pool of two flies from IAEA colony.

from Austria had about half as much KI 3150 (0.2 µg/female) as KI 3350 and KI 3550 (0.37 µg/female of each).

Mass spectrometry of alkenes

Treatment of small portions of the alkene fraction (∼100 females) with deuterium or dimethyl disulphide (Carlson *et al.*, 1989) yielded derivatives that survived GC/MS conditions, and showed clearly that one double bond was present on the shorter terminal end of each alkene in the three major alkenes.

A portion of this alkene fraction was deuterated with palladium/charcoal catalyst to yield hydrocarbons in which deuterium (D_2) had been added across sites of unsaturation. Mass spectrometry clearly showed that deuterium was present on the short end of C27 or larger alkenes. Fragmentation patterns showed ion clusters consistent with D_2 addition centred at *m/z* 169–171, *m/z* 197–201 and *m/z* 267–272 for KI3150D2, KI 3350D2 and KI 3550D2, respectively (Figs 1a, 2a and 3a). Fragments without D_2 from the longer ends were entirely consistent with fragment ions derived from the longer ends of the corresponding natural alkanes, KI 3155 (C14CCH3-, *m/z* 224/225; C21, *m/z* 297), KI 3355 (C16CCH3-, *m/z* 252/253; C23, m/z 323) and KI 3355 (C18CCH3-, *m/z* 280/281; C25, *m/z* 355) (Carlson *et al.*, 2000).

A second portion of this alkene fraction, to which dimethyl disulphide had been added, showed fragmentation of the carbon–carbon bond between the added methyl sulphide adducts, and gave one major fragment ion for each major alkene (KI 3150, *m/z* 495; KI 3350, *m/z* 524; KI 3550, *m/z* 552 in which the mass defect added 1 amu) (Figs 1b, 2b and 3b), respectively. The small one-carbon ion fragments could not be detected, as expected. The adduct ions observed at *m/z* M +47 and M +94 confirmed the total number of carbons for each homolog. Combining structural information derived from the dimethyl disulphide adducts and the D_2 homologs, the major alkenes from females had two methyl branches consistent with structures 1,2-dideutero-13,17-dimethyltritriacontane and 1,2-dideutero-13,17-dimethylpentatriacontane. Thus, the natural alkenes from females were deduced to be 13,17-dimethyltritriacont-1-ene (KI 3350) and 13,17-dimethylpentatriacont-1-ene (KI 3550), as no evidence was seen that other methyl-branched positional isomers were present.
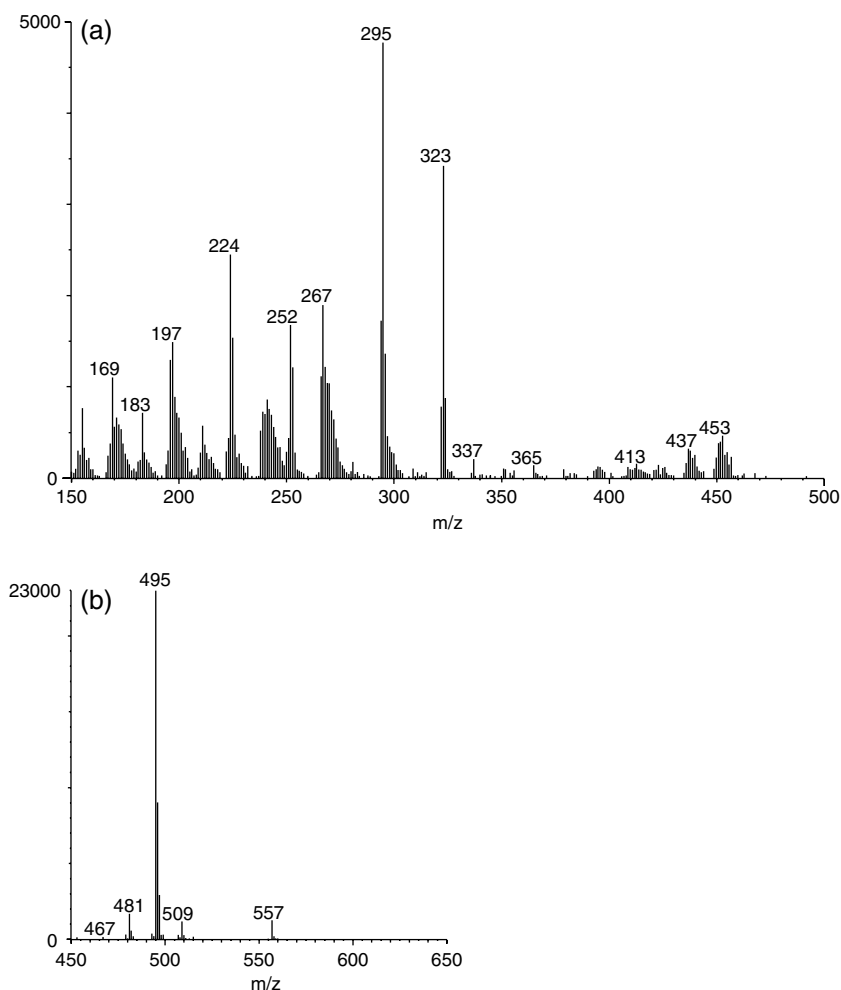

Fig. 1. Analysis by gas chromatography–mass spectrometry (GC–MS) of separated natural alkene KI 3150 from females separated by preparative GC. (a) Deuterated adduct, (b) dimethyl disulphide adduct.
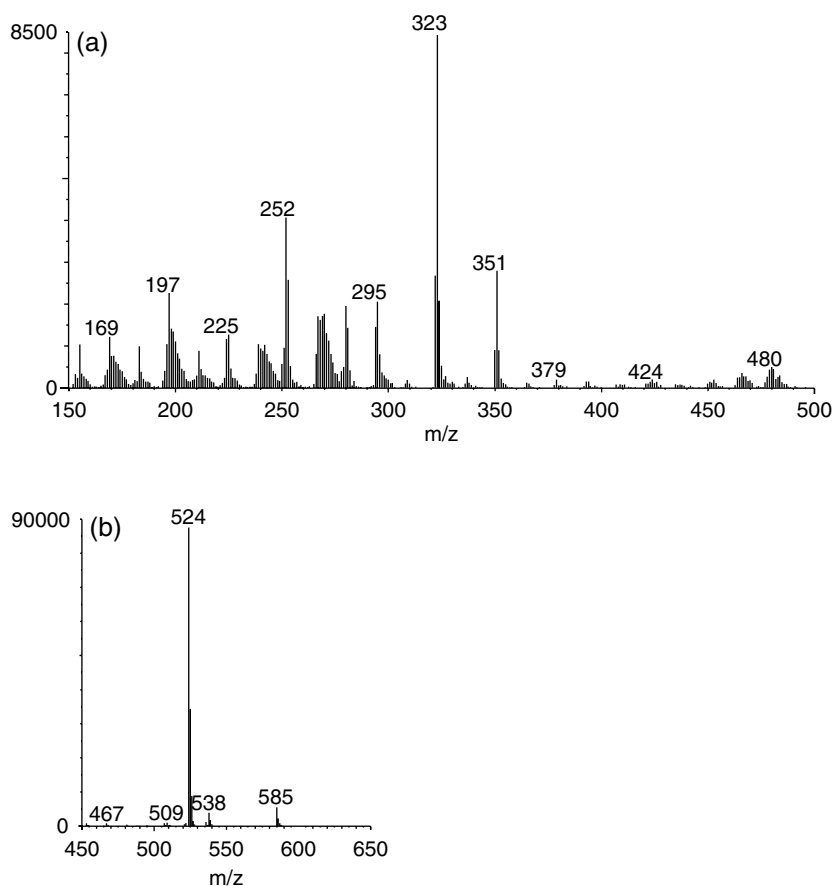
Fig. 2. Analysis by gas chromatography–mass spectrometry (GC–MS) of separated natural alkene KI 3350 from females separated by preparative GC. (a) Deuterated adduct, (b) dimethyl disulphide adduct.

One dimethylalkene was present in minor amounts in the peak eluting at KI 2550 (9,13-dimethylpentacos-1-ene), but there were no other isomers as only a C12 unsaturated fragment was observed. For the KI 2750 alkene isomers, two unsaturated fragment ions were observed, suggesting about four times more of the isomer with a longer (C12) unsaturated end than the shorter end (C10). Males contained the alkenes 9,13- and 11,15-dimethylnonacos-1-ene (KI 2950) and a major mixture of 13,17- and 11,15-dimethylhentriacont-1-ene (KI 3150, data not shown). Small amounts of the even-carbon backbone alkenes 14,18- and 12,16-dimethyldotriacont-1-ene (KI 3250) and 14,18- and 12,16-dimethyltetratriacont-1-ene (KI 3450) were seen in females. The structures found in smaller female-produced mono-alkenes were consistent with structures of the dominant alkenes, having the terminal double bond on the C10 and C12 end of the molecule.

Behavioural studies: responses to natural hydrocarbons

Males responded well to dead unwashed females, but never responded to solvent-washed decoys. Bioassays conducted in Seibersdorf, Austria in 1994 showed that laboratory males exhibited sexual behaviour in dose–response studies of the total hydrocarbons at 10 female equivalents.

Full copulatory responses were also observed in Austria with the alkene fraction obtained from conspecific females, at doses as low as one female equivalent, assumed from previous work to be 10 µg.

Results from the dose–response tests in Tanga, which used hydrocarbons that had been separated into total alkanes and total alkenes, showed that the alkenes were mostly responsible for this activity at about 10 µg or more (Table 3). However the total alkane fraction did not yield a full sexual response at any dose.

Bioassays in Tanga by two independent workers (F.M. and K.M., three trials at each quantity) tested five natural alkenes and five alkanes that had nearly the same retention times on the GC columns used (Table 4). These were collected by preparative GC and quantified by analytical GC before testing. The earlier eluting major alkene, KI 3350, showed responses of 70%, 66% and 100% in tests of 0.5, 1 and 5 µg, respectively, by F.M. compared to 66% with the control. The later-eluting alkene, KI 3550, showed 21% and 40% average response compared to 84% for control females. Three of these treatments obtained more copulatory responses than unwashed dead test females. Curiously there were no responses in tests by K.M. of this peak, but the data were included.

All of the alkenes tested elicited a significantly stronger behavioural response from males than was observed with solvent-washed decoys (Table 5). A dose–response
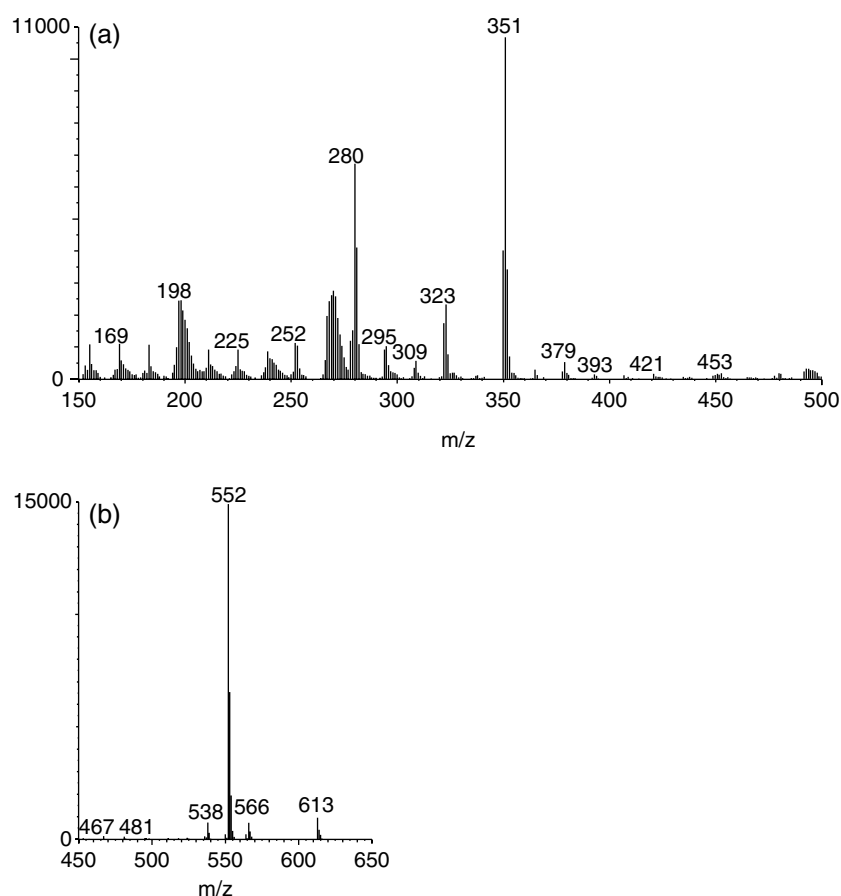
Fig. 3. Analysis by gas chromatography–mass spectrometry (GC–MS) of separated natural alkene KI 3550 from females separated by preparative GC. (a) Deuterated adduct, (b) dimethyl disulphide adduct.

Table 3. Sexual response bioassays of mature *Glossina austeni* males to conspecific decoy females dosed with different quantities of female *G. austeni* total alkane and alkene fractions. Ten males tested against each of 10 decoys at each dose

Material tested	µg tested	No. of males responding at each RL*			
		1	2	3	4
Alkanes	0.0	10	0	0	0
	1.0	10	0	0	0
	2.0	0	10	0	0
	5.0	0	10	0	0
	10.0	0	10	0	0
	25.0	0	10	0	0
	50.0	10	0	0	0
Alkenes	0.0	10	0	0	0
	1.0	10	0	0	0
	2.0	0	10	0	0
	5.0	0	10	0	0
	10.0	0	0	0	10
	25.0	0	0	0	10
	50.0	0	0	0	10

*Score for RL1 = brief contact, RL2 = mount and orientation, RL3 = curving abdomen, RL4 = hypopygium extension.

relationship was observed in responses of males to decoys treated with homologs KI 3350 and KI 3550, with maximum responses (score range 3.2–3.5) at the high doses of 25 and 50 µg. There were no significant differences in responses to decoys treated with the same doses of homologs KI 3350 and 3550, but both were significantly different from the three other earlier-eluting homologous peaks.

Responses of males to decoys treated with alkanes were only slightly higher than responses to controls for all homologs except KI 3555 (Table 4). KI 3555 elicited significantly stronger responses than the other homologs, with mean scores of 2.5 and 2.2, at 2 and 5 µg doses, respectively. Responses to KI 3555 were also numerically stronger than other homologs at 25 and 50 µg but the differences were not statistically significant. This is not surprising, as the synthetic racemic alkane 13,17-dimethyltritriacontane gave excellent responses to males of this species in early U.K. laboratory tests (Huyton *et al.*, 1980). It appears that the alkanes tested here are behaviourally bioactive, but this was not well quantified.

Dose–response to synthetic alkenes

Following the structural studies that were partially reported in Carlson *et al.* (2000), four stereoisomers of

Table 4. Sexual response bioassays of *Glossina austeni* males to dead male washed decoys and to decoys dosed with preparative gas chromatography-collected peaks of natural alkenes and alkanes. (n = 10)

| | ME* | Mean (SE) response score to doses of 1–50 µg | | | | | | ANOVA F† | P-value |
		1	2	5	10	25	50		
Alkenes									
2980	0.0 (0.0)[b]	1.0 (0.0)[a]	1.0 (0.0)[a]	1.0 (0.0)[a]	1.5 (0.5)[a]	1.0 (0.0)[aB]	1.0 (0.0)[aB]	39.0	<0.0001
3060	0.0 (0.0)[b]	1.0 (0.0)[a]	1.0 (0.0)[a]	1.3 (0.3)[a]	1.3 (0.3)[a]	1.2 (0.0)[aB]	1.0 (0.0)[aB]	5.8	0.0003
3160	0.0 (0.0)[b]	1.0 (0.0)[a]	1.0 (0.0)[a]	1.0 (0.0)[a]	1.2 (0.2)[a]	1.5 (0.5)[aB]	1.0 (0.0)[aB]	10.2	<0.0001
3350	0.0 (0.0)[d]	1.0 (0.0)[cd]	1.0 (0.0)[cd]	1.7 (0.5)[bcd]	2.2 (0.6)[abc]	3.2 (0.5)[abA]	3.5 (0.5)[aA]	9.6	<0.0001
3550	0.0 (0.0)[c]	1.0 (0.0)[bc]	1.0 (0.0)[bc]	1.7 (0.5)[b]	1.5 (0.0)[bc]	3.3 (0.5)[aA]	3.5 (0.5)[aA]	1.6	<0.001
ANOVA F‡	–	–	–	0.93	1.35	8.36	18.75		
P-value	–	–	–	0.46	0.28	0.0002	<0.001		
Alkanes									
2985	0.2 (0.2)[b]	1.0 (0.0)[ab]	1.0 (0.0)[abB]	1.0 (0.0)[abB]	1.5 (0.5)[a]	1.0 (0.0)[ab]	1.0 (0.0)[ab]	3.9	0.0044
3065	0.0 (0.0)[b]	1.0 (0.0)[a]	1.2 (0.2)[aB]	1.0 (0.0)[aB]	1.0 (0.0)[a]	1.0 (0.0)[a]	1.5 (0.5)[a]	5.3	0.0006
3165	0.2 (0.2)[b]	1.0 (0.0)[a]	1.0 (0.0)[aB]	1.2 (0.2)[aAB]	1.5 (0.5)[a]	1.0 (0.0)[a]	1.0 (0.0)[a]	5.0	0.0009
3355	0.0 (0.0)[b]	1.0 (0.0)[a]	1.0 (0.0)[aB]	1.0 (0.0)[aB]	1.3 (0.3)[a]	1.0 (0.0)[a]	1.0 (0.0)[a]	11.0	<0.0001
3555	0.2 (0.2)[b]	1.0 (0.0)[ab]	2.5 (0.7)[aA]	2.2 (0.5)[aA]	1.5 (0.5)[a]	2.3 (0.6)[a]	2.2 (0.6)[a]	3.3	0.0113
ANOVA F	1.0	–	4.04	4.01	0.52	2.7	2.2		
P-value	0.426	–	0.0116	0.0120	0.720	0.054	0.103		

*ME dose is unwashed dead male decoy: over all tests, no statistical differences were observed between unwashed decoys.
†ANOVA F and P-values at end of each row are for comparisons of doses within peaks. Means within rows followed by the same lower case letter are not significantly different at $P = 0.05$ (Duncan's multiple range test). Range tests were only done if the ANOVA F was significant.
‡ANOVA F and P-values at end of each column (within C33 or C35 series) are for comparisons of peaks within doses. Means within columns followed by the same upper case letter are not significantly different at $P = 0.05$ (Duncan's multiple range test).

each of the major alkenes were synthesized and made available for the present bioassays (Kimura *et al.*, 2001). Dose–behavioural response data were analysed for males to the four stereoisomers of 13,17-dimethyltritricont-1-ene. The percentage of males that gave full copulatory responses to treated decoys, dead washed males, and dead female controls was used. Responses to unwashed female decoys run as positive controls compared over all tests showed no statistical between-test differences in response (Table 5).

Table 5. Sexual response bioassays of *Glossina austeni* males to dead female decoys and to decoys dosed with individual synthetic stereoisomers of 13,17-dimethyltritricont-1-ene and 13,17-dimethylpentatriacont-1-ene. (n = 6)

| Synthetic pheromone | FE* | Mean RL (SE) scores to doses of 1–50 µg | | | | | | ANOVA F† | P-value |
		1	2	5	10	25	50		
C33:1 *R,R*	2.50 (0.67)	1.00 (0.00)	1.83 (0.54)	1.50 (0.50)	1.00 (0.00)	1.00 (0.00)[B]	1.00 (0.00)[B]	1.43	0.2426
C33:1 *R,S*	2.00 (0.16)	1.16 (0.16)	1.66 (0.33)	2.00 (0.36)	1.83 (0.54)	1.83 (0.54)[B]	2.00 (0.51)[AB]	0.52	0.7611
C33:1 *S,R*	3.83 (0.16)	1.50 (0.50)	2.00 (0.63)	2.00 (0.63)	2.00 (0.63)	3.33 (0.49)[A]	3.33 (0.49)[A]	1.87	0.1292
C33:1 *S,S*	3.00 (0.63)	1.33 (0.33)[bc]	1.33 (0.21)[bc]	1.00 (0.00)[c]	2.33 (0.61)[ab]	1.00 (0.0)[cB]	2.50 (0.50)[aA]	3.38	0.0153
ANOVA F‡		0.48	0.38	1.17	1.20	8.99	5.00		
P-value		0.7024	0.7676	0.3459	0.3352	0.0006	0.0095		
C35:1 *R,R*	3.57 (0.42)	1.00 (0.00)	1.66 (0.42)	2.00 (0.51)	1.16 (0.16)	1.00 (0.00)[B]	1.50 (0.22)	1.87	0.1289
C35:1 *R,S*	3.16 (0.54)	1.16 (0.16)[b]	1.00 (0.00)[b]	1.33 (0.33)[b]	1.83 (0.40)[ab]	2.33 (0.49)[aA]	1.16 (0.16)[b]	2.73	0.0380
C35:1 *S,R*	4.00 (0.00)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)	1.16 (0.16)	1.00 (0.00)[B]	1.33 (0.21)	1.62	0.1863
C35:1 *S,S*	3.50 (0.50)	1.50 (0.50)	1.50 (0.50)	1.50 (0.34)	1.66 (0.33)	1.16 (0.16)[B]	1.66 (0.16)	0.32	0.8975
ANOVA F		0.80	1.10	1.40	1.44	6.09	0.68		
P-value		0.5084	0.3708	0.2707	0.2607	0.0041	0.5751		

*FE dose is unwashed dead female decoy: over all tests, no statistical differences were observed between unwashed decoys.
†ANOVA F and P-values at end of each row are for comparisons of doses within peaks. Means within rows followed by the same lower case letter are not significantly different at $P = 0.05$ (Duncan's multiple range test). Range tests were only done if the ANOVA F was significant.
‡ANOVA F and P-values at end of each column (within C33 or C35 series) are for comparisons of peaks within doses. Means within columns followed by the same upper case letter are not significantly different at $P = 0.05$ (Duncan's multiple range test).

When scores for the positive controls were omitted, there was no statistical significance between response scores treated with increasing doses for *R,R*- and *R,S*-33:1, despite trends suggesting that *S,R*- was more bioactive. The mean response scores to the *S,S*-33:1 stereoisomer at 50 µg were significantly higher than responses to all other doses except 10 µg.

In tests with the 35:1 stereoisomers, there were no significant differences in response among doses with the exception of *R,S*-C35:1. Responses to the latter at 25 µg were significantly stronger than to all other doses except 10 µg.

When responses were compared across 33:1 stereoisomers at the same dose, no significant differences were observed at 1, 2, 5, or 10 µg. At 25 µg, responses to the stereoisomers were significantly stronger to *S,R*- than to the others. At 50 µg, responses to *R,R*- were significantly lower than to *S,R*- or *S,S*-, possibly signifying an overdose. Responses to *S,R*- were higher than the other stereoisomers, but no statistical difference was observed among *R,S*-, *S,R*- and *S,S*-. In tests with C35:1 stereoisomers, responses to *R,S*- were significantly stronger than the other three stereoisomers at 25 µg, and no significant responses were observed at the other doses.

Discussion

Presence of alkanes and alkenes in different populations

Glossina austeni occur in many geographically distinct populations in Africa, but these populations are similar in the composition of their surface hydrocarbons including specific sex pheromones. Alkanes of females from seven locations were analysed to determine differences. Three groups could be seen: (1) Bristol laboratory, ILRAD/Kenya/Bristol, Maisons-Alfort/Bristol, (2) Vienna, Mozambique wild, Natal-Zululand-SA wild, Tanga laboratory, (3) Kenya wild. The absolute meaning of these differences is not known, as the biological activity of the alkanes that were observed was in response to doses that were 100–200 times the natural quantity.

Alkenes were observed to be structurally the same among all specimens examined. Laboratory evidence indicates that these alkenes do not exhibit the degree of temporal stability found in the methyl-branched alkanes, because they may be lost after chemical separation from alkanes in the laboratory on silver nitrate-impregnated silica gel. The alkenes apparently undergo oxidation and can no longer be found intact by GC analysis in old separated samples (Carlson, unpublished data). Thus, more recent collections of *G. austeni* from Kenya and elsewhere are needed to confirm these observations, and to show whether significant geographical variation in contact sex pheromone components may occur in this species. Also, it would be interesting to know whether the major C31:1 alkene present in virgin males and found in large amounts on mated females has a

negative effect on mating success in colonies, but this synthetic alkene is not available for bioassays.

Activity of synthetic stereoisomers

This is the first example in tsetse that sexual activity was released by a synthetic alkene. Full copulatory attempts were observed here during a number of tests of the lower doses of the synthetic compounds. Results of bioassays of the stereoisomeric alkenes showed excellent responses of males to two compounds, *S,R*-C33:1 and *R,S*-C33:1. A dose–response relationship was seen for the *S,R*-stereoisomer in which the response increased from 33% to 66% at lower doses, and up to 73% at 25 µg, somewhat lower than responses to the control at 83%. Over all tests, the responses averaged ~3/4 of those to the control. Responses to the *R,S*-compound averaged ~2/3 of those to the control. The other two C33:1 stereoisomers, *R,R*- and *S,S*-, were ~1/3 and ~1/5 as active as the control. The response to one C35:1 stereoisomer, *R,R*-C35:1, was higher at 2 (25%) and 5 µg (36%) than at higher quantities. Trials of mixtures of mixed stereoisomers and field trials with wild flies will be reported separately.

Discovery of a sex pheromone may help in modern biocontrol efforts against this disease vector by ensuring that a competitive strain of fly is used in large-scale sterile male releases. Also, a synthetic pheromone might be used to increase the very slow rate of reproduction in laboratory-reared flies, and to test sexual behaviour in reared males intended for mass release.

We had observed previously that male *G. austeni* produced one major C31 alkene, whereas females produced primarily C33 and C35 alkenes (Carlson & Langley, 1986). It is reasonable to conclude that these three compounds are all congeners of each other, with the methyl branching and double bond in the same relative positions. The present results confirm this conclusion, when compared to older results, except that much more material (3–5 µg of these alkenes per female) was found to be present in recently collected females. Thus, it seems reasonable to deduce that the latter two compounds comprise the sexual stimulant in this species. Collections of flies from different locations including individual dried females showed consistency in these two alkenes.

Curiously, the minor trimethylalkanes described here are the most abundant alkanes present in females of other members of the Nemorhina group, *G. palpalis palpalis*, *G. palpalis gambiensis* and *G. fuscipes fuscipes*: Testing of male *G. austeni* against allospecific females showed that males did respond at 100% (20/20) to *G. palpalis palpalis* females and 100% (40/40) to *G. fuscipes fuscipes* females, just as they did to conspecific dead females at 100% (20/20) (Huyton *et al.*, 1980). This strongly suggests that individual natural and synthetic trimethylalkanes release sexual stimulant activity in *G. austeni*.

Clearly, the alkenes tested here were the most active materials in bioassays conducted in Africa. Much less

alkene material than alkanes was present on the cuticle of mature females. The most active natural component was KI 3350, which contained one asymmetric centre, but there appeared to be significant bioactivity in the KI 3550 peak as well as lesser activity in the corresponding natural alkane.

We conclude that the relatively few data available for evaluation here determined that the *R,S*-, *S,R*- and *S,S*-C33:1 stereoisomers were the most bioactive, with *S,R*- scoring the best statistically. Bioassays showed that the synthetic *S,R*-C33 stereoisomer evoked the same sexual stimulation in test males as a dead female fly, and nearly the same numbers of responses as a dead female fly decoy. Although more trials are needed to define the activity, this stereoisomer represented very well the natural pheromone of the tsetse fly *G. austeni*. A combination of the best C33:1 stereoisomers or addition of a C35:1 stereoisomer may be necessary to obtain lower active dose and treatment scores, but that was beyond the scope of the present effort.

Acknowledgements

References

Carlson, D.A. & Langley, P.A. (1986) Tsetse alkenes: appearance of novel sex-specific compounds as an effect of mating. *Journal of Insect Physiology*, **32**, 781–790.

Carlson, D.A., Langley, P.A. & Huyton, P.M. (1978) Sex phero- mone of the tsetse fly: isolation, identification and synthesis of contact aphrodisiacs. *Science*, **201**, 750–753.

Carlson, D.A., Nelson, D.R., Langley, P.A., Coates, T.W., Davis, T.L. & Leegwater-Vander Linden, M. (1984) Contact sex pher- omone in the tsetse fly *Glossina pallidipes* (Austen); identifica- tion and synthesis. *Journal of Chemical Ecology*, **10**, 429–450.

Carlson, D.A., Roan, C.-S., Yost, R.-A. & Hector, J. (1989) Dimethyl disulfide derivatives of long chain alkenes, alkadienes, and alkatrienes for gas chromatography/mass spectrometry. *Analytical Chemistry*, **61**, 1561–1571.

Carlson, D.A., Milstrey, S.K. & Narang, S.K. (1993) Genetic classification of the tsetse flies using cuticular hydrocarbons. *Bulletin of Entomological Research*, **83**, 507–515.

Carlson, D.A., Offor, I.I., El Messousi, S., Matsuyama, K., Mori, K. & Jallon, J.-M. (1998) Sex pheromone of *Glossina tachi- noides*: identification and synthesis. *Journal of Chemical Ecology*, **24**, 1563–1575.

Carlson, D.A., Sutton, B.D. & Bernier, U.R. (2000) Cuticular hydrocarbons of *Glossina austeni* and *G. pallidipes*: Similarities between populations. *Insect Science and its Application*, **20**, 281– 294.

Huyton, P.M., Langley, P.A., Carlson, D.A. & Schwartz, M. (1980) Specificity of contact sex pheromones in tsetse flies, Glossina spp. *Physiological Entomology*, **5**, 253–264.

Kimura, T., Carlson, D.A. & Mori, K. (2001) Pheromone synth- esis, Part 211: Synthesis of all of the stereoisomers of 13,17- dimethyl-1-tritriacontene and 13,17-dimethyl-1-pentatriacon- tene: the contact sex pheromone components of the female tsetse fly, *Glossina austeni*. *European Journal of Organic Chemistry*, 3385–3390.

Kovats, E. (1965) Gas chromatographic characterization of organic substances in the retention index system. *Advances in Chromatography*, **1**, 229–247.

Kuwahara, K. & Mori, K. (1983) Synthesis of all three stereoi- somers of 13,23-dimethylpentatriacontane, the sex pheromone of the tsetse fly, *Glossina pallidipes*. *Agricultural and Biological Chemistry*, **47**, 2599–2606.

Langley, P.A., Pimley, R.W. & Carlson, D.A. (1975) Sex recogni- tion pheromone of the tsetse fly. *Nature*, **254**, 51–53.

McDowell, P.G., Hassanali, A. & Dransfield, R. (1985) Activity of the diastereomers of 13,23-dimethylpentatriacontane, the sex pheromone of *Glossina pallidipes*, and comparison with the nat- ural pheromone. *Insect Biochemistry*, **16**, 83–190.

Nelson, D.R. & Carlson, D.A. (1986) Cuticular hydrocarbons of the tsetse flies *Glossina morsitans*, *G. austeni* and *G. pallidipes*. *Insect Biochemistry*, **16**, 403–416.

Nelson, D.R., Carlson, D.A. & Fatland, C.L. (1988) Cuticular hydrocarbons of the tsetse flies, II: *G. p. palpalis*, *G. p. gambiensis*, *G. fuscipes*, *G. tachinoides* and *G. brevipalpis*. *Journal of Chemical Ecology*, **14**, 963–987.

Offor, I.I., Carlson, D.A., Gadzama, N.M. & Bozimo, H.T. (1981) Sex recognition pheromone in the West African Tsetse fly, *Glossina palpalis palpalis*. *Insect Science and its Application*, **1**, 417–420.

SAS (1992) *SAS Users Guide: Statistics*. SAS Institute, Cary, NC.

Sutton, B.D. & Carlson, D.A. (1997) Cuticular hydrocarbons of Glossina III: Subgenera Glossina and Nemorhina. *Journal of Chemical Ecology*, **23**, 1291–1320.